Chapter 17

Simultaneous Saccharification and Fermentation and Partial Saccharification and Co-Fermentation of Lignocellulosic Biomass for Ethanol Production

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Summary

Ethanol production by fermentation of lignocellulosic biomass-derived sugars involves a fairly ancient art and an ever-evolving science. Production of ethanol from lignocellulosic biomass is not avant-garde, and wood ethanol plants have been in existence since at least 1915. Most current ethanol production relies on starch- and sugar-based crops as the substrate; however, limitations of these materials and competing value for human and animal feeds is renewing interest in lignocellulose conversion. Herein, we describe methods for both simultaneous saccharification and fermentation (SSF) and a similar but separate process for partial saccharification and cofermentation (PSCF) of lignocellulosic biomass for ethanol production using yeasts or pentose-fermenting engineered bacteria. These methods are applicable for small-scale preliminary evaluations of ethanol production from a variety of biomass sources.

Key words: Saccharification, Fermentation, Lignocellulose, Pretreatment, GC

1. Introduction

Ethyl alcohol has been used by humans since the dawn of history and is thought to be one of the most universally known chemical compounds manufactured *(1)*. Produced by the spontaneous fermentation of sugars, ethanol was used by ancient civilizations that evolved many types of production. Some cultures extracted and concentrated alcohol in crude stills and used it in the manufacture of perfumes, cosmetics, medicinal agents, and beverages. Ethanol found increasing use as a chemical agent, an ingredient, or a raw material for the production of other commodities, as

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later civilizations improved upon the purification and distillation processes. With the entry of the United States into World War II, the alcohol requirements for munitions, synthetic rubber, solvents and thinners, and food increased the demand to unprecedented levels *(1)*.

Ethanol may be produced by fermentation using three main groups of feedstock: saccharine-containing materials (molasses, fruit, sugar cane juices, etc.); starchy materials (cereal grains, root crops such as potatoes, etc.); and cellulosic or lignocellulosic materials (wood, waste sulfite liquor from paper pulp mills, agricultural residues such as corn cobs, hulls, stover, etc.). Using lignocellulose for ethanol production is not novel, and considerable effort was invested in converting sawdust and mill waste to ethanol using a dilute sulfuric acid process as early as 1915 *(1)*. Ethanol was produced commercially in the United States by this method until the end of World War I, when molasses became a cheap source of readily available substrate.

Fermentation processes using wood wastes intensified abroad, however, since wood waste was more economically available than molasses or grain. By 1941, 21 foreign plants are reported to have operated on wood wastes. A commercial plant was erected for the development of ethanol from wood wastes in the United States by Defense Plants Corporation and was operational in 1947 *(1)*; however, further development of this technology ceased when wartime scarcity disappeared and the era of inexpensive petrochemical fuels began *(2)*.

Bioconversion of cellulosics was still pursued by the U.S. Army Natick Research and Development Command following WW II, but their interest pertained to protecting cellulosic materials used by the military (e.g., cotton uniforms) from microbial degradation *(3)*. These scientists isolated the dominant organism responsible for decomposition of military clothing, tents, and other equipment in 1943. The organism was the fungus *Trichoderma reesei*, now known as an anamorph of *Hypocrea jecorina(4)*. Hereafter, *T. reesei* is referred to as *H. jecorina*. Army researchers further identified an active cellulase complex that was stable and contained all the components needed to hydrolyze native cellulose. A hyper-secreting *H. jecorina* mutant was isolated in 1971, and in 1973 considerable effort focused on process development for production of cellulases and pretreatment options for various feedstock *(5)*. Current commercial processes for producing cellulase are based on modifications of this fungus and its relatives.

In 1974, Gulf Oil Chemicals Company undertook extensive research and development to examine processes that could convert cellulose to chemicals. Their objective was to establish a chemical industry that was based on a renewable resource rather than petroleum *(6)*. The subsequent OPEC oil embargo

emphasized the importance of developing this type of industry for obtaining greater energy independence. In 1976, a method was patented with yeast as the biocatalyst, which prevented glucose accumulation by combining the saccharification and fermentation steps, termed the simultaneous saccharification and fermentation (SSF) process ***(7, 8)***. Faster enzyme activity is maintained because the glucose is fermented as soon as it is released by cellulase, thereby minimizing end-product inhibition. The alternative process, i.e., separate hydrolysis followed by fermentation (SHF), generates a sugar stream first, followed by inoculation with the fermenting organism for conversion of the sugars to ethanol. Partial saccharification and cofermentation (PSCF) is a combination of SSF and SHF, whereby the enzymes (usually from fungi) are given a "head start" under optimum conditions (45–50°C and ≤pH 5) to liberate some of the available monomeric sugars from the biomass polymers. After a period of a few hours the conditions are altered for the fermenting organism inoculation; the pH is usually raised and temperature often lowered. In this fashion, the fermenting organism rapidly consumes the previously liberated sugars, and the enzymes are still able to work, albeit at reduced efficiency. True SSF requires a lower capital cost, generally produces higher concentrations of ethanol, and reduces risks from contamination because the accumulation of sugars is avoided ***(9)***. Selecting appropriate operating conditions of pH and temperature that favor both the enzymes and fermenting organism in an SSF process is crucial for maximizing yield and productivity, although there is some degree of flexibility in the range of both ***(10)***.

In the United States, almost all ethanol is produced by fermenting dent corn, which is 60–65% starch; starch is an α-1,4-linked polymer of glucose that is easily digested by humans or, in the case of ethanol production, by commercial amylases ***(11, 12)***. The starch is either converted to glucose by a two-step enzyme process involving liquefaction followed by saccharification, or by a newer one-step process using native starch amylases. In both processes, usually the final release of glucose and fermentation are carried out simultaneously. Fermentation substrates, such as starch, which have competing value for animal and human needs, will be insufficient to meet the increasing demands for fuel ethanol. Therefore, a more plentiful and less expensive source of carbohydrate is needed as the feedstock. Furthermore, lignocellulosic ethanol offers larger reductions in greenhouse gases compared to corn ethanol or petroleum-based fuels ***(13)***.

Cellulosic wastes, agricultural residues, and forage and woody crops are significant renewable resources for the production of fermentable sugars ***(14–18)***. Ethanol production by fermentation of lignocellulosic biomass-derived sugars involves a fairly ancient art and an ever-evolving science. In general, lignocellulose is treated

to open the plant wall structure and disrupt lignin–hemicellulose complexes *(19)*. Hemicellulose is a heterogeneous branched polymer that yields mostly xylose upon hydrolysis, as well as some arabinose, mannose, glucose, galactose, acetic acid, glucuronic acid, and furfural, depending upon the biomass type. Hydrolysis of the hemicellulose component to yield hexoses and pentoses is relatively easy compared to cellulose hydrolysis; however, efficient cofermentation of hexose and pentose sugars presents a challenge. Conversely, fermentation of the cellulose hydrolysis product, i.e., glucose, is straightforward, while the actual hydrolysis step itself is more difficult *(20, 21)*. In its native form, cellulose is composed largely of crystalline fibers held together by an extensive network of hydrogen bonds. These fibers are embedded in a matrix of hemicellulose and lignin, which serves to further reduce their accessibility to cellulolytic enzymes *(22, 23)*. Solvent and mechanical pretreatments increase the accessibility of cellulose to hydrolysis presumably by disrupting the lignin matrix and crystalline structure of the cellulose. Therefore, pretreatment of lignocellulosics improves cellulose conversion *(19, 21, 24)*. Pretreatment methods that have been examined for biomass conversion to ethanol include acid pre-hydrolysis, steam explosion, ammonia fiber expansion (AFEX), alkali treatment, organic solvents, and radiation, as well as numerous others *(2, 11, 12, 25, 26)*. Many of the pretreatment methods, in addition to separating the cellulose, hemicellulose, and lignin, also hydrolyze the hemicellulose to monosacchrides. In contrast, cellulose must be converted to either glucose or cellobiose before fermentation, depending upon the fermenting microorganism's ability to use dimers of glucose versus the monomeric form. Lignin, the third major component of lignocellulose, is a large phenolic polymer that cannot be fermented to ethanol but when combusted can provide sufficient energy for ethanol recovery *(26)*.

The enzymatic hydrolysis of cellulose is particularly attractive because of its mild reaction conditions, high selectivity, and low impact on the environment *(19, 20, 27)*. The complete hydrolysis of cellulose to glucose requires at least three major classes of enzymes: (1) exoglucanases, which attack nonreducing ends of crystalline cellulose chains; (2) endoglucansases, which degrade amorphous cellulose and may also introduce nicks in crystalline cellulose chains; and (3) β-glucosidase (cellobiase), which completes the process by degrading cellobiose into glucose monomers *(20, 28)*. Deconstruction of intact plant cell walls is much more complex and involves many additional enzymatic activities. For example, enzymes such as phenolic esterases break the bonds between some carbohydrates and lignin and can enhance digestibility *(29, 30)*. If hemicellulose is to be enzymatically digested, an additional suite of activities is required. For this discussion, hemicellulose is hydrolyzed with acid to liberate xylose, and enzymes are used to convert cellulose to glucose.

Microorganisms that degrade cellulose are ubiquitous and abundant in nature. These include fungi, bacteria, and actinomycetes. The ability to produce extracellular cellulolytic enzymes is widespread among fungi, with *Hypocrea jecorina* being one of the most extensively studied. Culture filtrates from this organism contain each of the major cellulolytic enzymes in a number of forms. While a multiplicity of each of these three major components exists, the mode of action of each general group of enzymes can be summarized for fungal enzymes as follows ***(28)***: (a) endoglucanase; (b) β-glucosidase; (c) cellobiohydrolase. Endoglucanases (EC 3.2.1.4) hydrolyze β-1,4- glycosidic linkages randomly and do not attack cellobiose. Most reports indicate minimal action on crystalline cellulose. Endoglucanases hydrolyze cellodextrins, phosphoric-acid-swollen cellulose, and substituted celluloses (indicating low specificity). The β-glucosidase enzyme (cellobiase, EC 3.2.1.21) hydrolyzes cellobiose and cello-oligosaccharides to glucose and does not attack cellulose or higher cellodextrins. Cellobiohydrolase (exocellulase, EC 3.2.1.91) splits off cellobiose units from the nonreducing end of the chain, does not attack substituted celluloses, and hydrolyzes cellodextrins, but not cellobiose. An extensive discussion of plant cell walls and their deconstruction is beyond the scope of this chapter on methods, and the reader is referred to a special edition of the plant journal: *Harnessing Plant Biomass for Biofuels and Biomaterials* (May 2008) and references therein for a more thorough discussion.

This chapter describes methods for dilute acid pretreatment followed by SSF and PSCF of biomass to produce ethanol. The methods encompass bacterial and yeast fermentations in either test tubes or customized bioreactors equipped with automatic pH control. These methods may be modified for a variety of biomass types. Mixed forest residues containing both 5C and 6C sugars were selected to illustrate results obtained via small volume or bioreactor fermentations using either yeast or engineered bacteria as biocatalyst. An overview of the process is presented in **Fig. 1.**

2. Materials

2.1. General Equipment

1. Countertop multi-stir water baths (model no. 1286Q, Barnstead Lab-Line, Dubuque, IA).
2. pH electrodes (Sensorex S300C, Sensorex Corp., Garden Grove, CA).
3. pH controllers (Jenco 3671, Jenco Instruments Inc., San Diego, CA).
4. Magnetic stir bars.

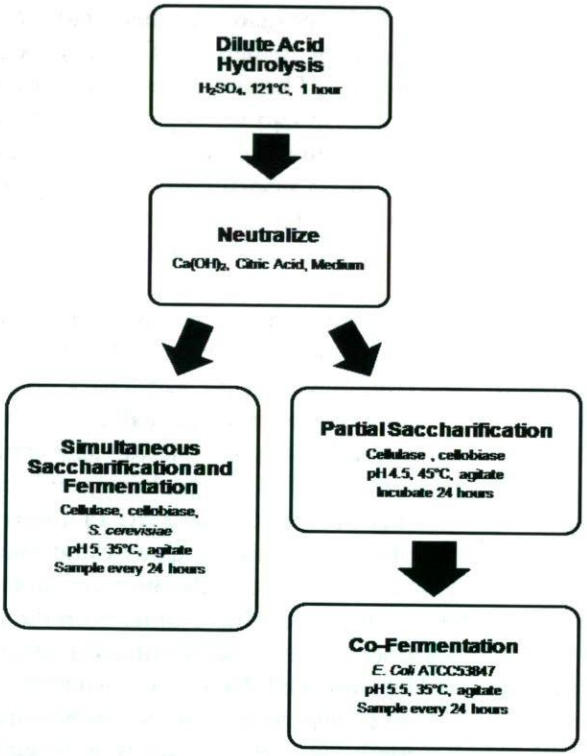

Fig. 1. Flowchart depicting the pretreatment and fermentation procedures for dilute acid hydrolysis and fermentation by *S. cerevisiae* and *E. coli* ATCC 53847. *See* **Subheadings 3.5–3.8** for details.

2.2. Chemical Solutions (All of Analytical Grade)

1. 2 M KOH.
2. 2 N HCl.
3. pH buffers.

2.3. Microorganisms

1. *Saccharomyces cerevisiae* D5A (ATCC No. 200062, National Renewable Energy Laboratory, Golden, CO) or other suitable *S. cerevisiae* strain (i.e., NABC BioFerm XR, North American Bioproducts, Norcross, GA).
2. Pentose and hexose fermenting bacterium such as *Escherichia coli* DC863adhc adhR, ATCC 53847 ***(31)***.

2.4. Media for Growth of Microorganisms

Unless otherwise directed, all solutions should be autoclaved for 20 min at 121°C on liquid cycle.

2.4.1. Saccharomyces cerevisiae

1. 10× YP Broth: 200 g peptone, 100 g yeast extract per liter of (distilled water) dH_2O.
2. Tryptic Soy Agar (TSA) plates: 15 g pancreatic digest of casein, 5 g papaic digest of soybean meal, and 5 g NaCl with 15 g agar per liter of dH_2O.

3. Glucose stock solution (50%, w/v), glucose and dextrose should be filter-sterilized or autoclaved separately from the medium and added to final concentrations indicated below to sterilized YP broth.
4. YP2D broth: 10 g yeast extract, 20 g peptone, 40 g dextrose, per liter of dH_2O.
5. YP5D broth: 10 g yeast extract, 20 g peptone, 100 g dextrose, per liter of dH_2O.
6. 1× Diluent: 4.25 g NaCl, 0.15 g KH_2PO_4, 0.3 g Na_2HPO_4, 0.2 g peptone, 500 ml dH_2O.

2.4.2. Escherichia coli

1. Modified Luria–Bertani (LB) broth: 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl per liter dH_2O.
2. 5× LB broth: 12.5 g in 100 ml dH_2O.
3. Glucose stock solution (50%, w/v).
4. 1× Diluent: 4.25 g NaCl, 0.15 g KH_2PO_4, 0.3 g Na_2HPO_4, 0.2 g peptone, 500 ml dH_2O.
5. Chloramphenicol stock solution: 400 mg chloramphenicol dissolved in 10-ml of 70% ethanol and filter-sterilized through a 0.22-μm filter.
6. LB/Gluc/CAM plate: LB agar plate with the following added after LB agar is cooled from autoclaving: 50% glucose stock solution for a final concentration of 2% w/v glucose, and 40 mg/ml chloramphenicol stock solution for a final concentration of 40 μg/ml.

2.5. Commercial Enzymes

1. GC 220 Cellulase (Danisco, Genencor Division, Rochester, NY) or Celluclast 1.5 l (Novozymes, Franklinton, NC).
2. Novo 188 Cellobiase (Novozymes, Franklinton, NC).
3. Pectinase from *Aspergillus niger* (P2736, Sigma Chemicals, St. Louis, MO).

2.6. Moisture Determination

1. Convection drying oven able to control heat at 105 ± 3°C.
2. Analytical balance, accurate to 0.1 mg.
3. Desiccator and desiccant.
4. Instead of **steps 1–3** above, an automated infrared moisture analyzer may be used.

2.7. Dilute Acid Pretreatment via Autoclaving and Fermentation of Biomass

1. Glass screw-top centrifuge tubes (50 ml), heat resistant above 121°C.
2. H_2SO_4 (1.75%, w/v).
3. $Ca(OH)_2$ (10%, w/v). $Ca(OH)_2$ will not dissolve; solution should be well mixed when using for additions.
4. Citric Acid [$HOC(COOH)(CH_2COOH)_2$] (1 M).

2.8. Fermentation of Biomass in Bioreactors

1. Glass bioreactors (250 ml).
2. Pasteur pipettes.
3. Pipetteman 200 µl tips.
4. Cotton.
5. Silicone lubricant.
6. 2× Tryptic soy broth (TSB).
7. 2× LB broth.
8. Aluminum foil.

2.9. Gas Chromatographic Analysis of Ethanol Concentration of Fermentation Samples

1. Gas chromatograph (Shimazdu GC-8A, Columbia, MD, or other equivalent GC) with a flame ionization detector.
2. Gas chromatograph column (J & W Scientific, 0.53 mm ID × 30 m, 3 µm film).
3. N_2, H_2, and compressed air gas cylinders.
4. Isopropanol (2%, v/v) internal standard in a stoppered volumetric flask.
5. Ethanol standards (0.5%, 1%, 2%, 3%, and 4%, v/v) in stoppered volumetric flasks.
6. Sample syringe (Hamilton syringes no. 80300), or autosampler.
7. Centrifugation filters (0.22 µm; Costar 8169, Spin-X centrifuge tube filter).

3. Methods

3.1. Moisture Determination of Biomass to be Fermented

1. Perform the moisture determination in triplicate (*see* **Note 1**).
2. Place an aluminum weigh boat into the convection oven at 105 ± 3°C for 4 h. Cool in a desiccator and weigh to the nearest 0.1 mg. Record.
3. Mix sample well, remove a representative sample, and weigh 1–2 g to the nearest 0.1 mg. Add to the predried weigh boat.
4. Place the sample into the convection oven at 105 ± 3°C for a minimum of 4 h. Very wet samples will require overnight drying. Cool the sample in a desiccator and weigh to the nearest 0.1 mg. Record. Determine whether the sample is at constant weight by reheating the sample for 1 h, drying in the desiccator, and reweighing. A 0.1% or less change in the weight is considered a constant weight.
5. To calculate the percent moisture, use the formula (initial weight–final weight)/initial weight) × 100.
6. Alternatively, an infrared moisture balance may be used to determine the moisture content.

3.2. Biomass Calculations

1. To determine percent solids of biomass, use: 100 – percent moisture.
2. To determine amount of wet biomass needed per culture to achieve a targeted dry weight, use: (y g dry wt)/(percent solids ÷ 100), where y is the total g sample on a dry weight basis required for fermentation.

3.3. Enzyme Calculations and Preparation

3.3.1. To Determine Amount of Commercial Enzymes in U (Units) Per Gram Dry Weight Biomass to Add to Fermentation

1. Determine total unit (U) of enzyme needed for the total volume of the fermentation and express the enzyme U for the total g dry weight of sample as: (g dry weight) × (U enzyme/g dry weight).
2. Determine total volume of commercial enzyme preparation to add to fermentation as: (total U enzyme calculated above)/(U/ml of commercial enzyme preparation).
3. Filter-sterilize enzymes using a 0.22-μm filter.

3.4. Microorganism Precultures

3.4.1. Saccharomyces cerevisiae

1. From –80°C freezer stock, streak *S. cerevisiae* D5A onto a tryptic soy agar (TSA) plate; incubate for 2 days at 37°C.
2. Inoculate YP2D broth (5–15 ml) with a single colony from the TSA plate; incubate overnight at 35°C with stirring at 150 rpm (*see* **Note 12**).
3. Transfer 1% (v/v) of overnight YP2D culture to YP5D medium; incubate for 24 h at 35°C at 150 rpm.
4. Measure the optical density at 600 nm (OD_{600}). Prepare the appropriate amount of culture for a final $OD_{600} = 0.5$. Centrifuge the culture, resuspend in 1× diluent, and add to the SSF.

3.4.2. Escherichia coli

1. From –80°C freezer stock, streak *E. coli* ATCC 53847 onto an LB plate containing 2% (w/v) glucose and 40 μg/ml chloramphenicol; incubate overnight at 37°C.
2. Inoculate 100 ml of LB broth containing 5% (w/v) glucose and 40 μg/ml chloramphenicol with a single colony from the LB plate; incubate overnight at 37°C without stirring (*see* **Note 12**).
3. Measure the OD_{550}. Prepare the appropriate amount of culture for a final $OD_{550} = 1$. Centrifuge culture, resuspend in 2× LB, and add to the fermentation.

3.4.3. Determining the Amount of Preculture Needed to Provide an Inoculum of OD 1

1. Use the following formula: $C_1V_1 = C_2V_2$, or, substituted, (OD_z preculture) (x ml) = (OD_z 1) (y ml), where x = amount of preculture needed, y = volume of fermentation, and z = wavelength.
2. Measure the volume of preculture needed and place in a centrifugation tube or bottle.
3. Centrifuge at 10,000 × g for 10 min and remove the supernatant.
4. Resuspend cells in a small volume of 1× diluent YP or LB (depending on fermentation method) and use to inoculate.

3.5. Small-Volume Dilute acid Pretreatment of Biomass by Autoclaving and Fermentation by Saccharomyces cerevisiae (Fig. 1)

3.5.1. Dilute Acid Hydrolysis by Autoclaving

1. Measure 1.5 g dry weight of biomass into 50-ml glass screw-top centrifuge tubes.
2. Add 8.5 ml 1.75% (w/v) sulfuric acid (density of concentrated H_2SO_4 = 1.84 g/ml)
3. Autoclave sealed tubes at 121°C for 1 h, and allow tubes to cool to room temperature.
4. Add 1.2 ml $Ca(OH)_2$, 0.55 ml 1 M citric acid, 1.1 ml 10× YP to each tube and adjust the pH to 5 (*see* **Notes 2** and **3**).

3.5.2. Enzyme Saccharification, Inoculation, and Fermentation

1. Prepare enzymes as directed above for an enzyme loading of 5 FPU cellulase/g dry weight biomass and 60 U cellobiase/g dry weight biomass (*see* **Note 7**).
2. Add enzymes to pretreated biomass tubes and incubate at 37°C at 150 rpm.
3. Prepare the inoculum as directed above (*see* **Subheading 3.4.1**) with 1× diluent.
4. Add the inoculum to pretreated tubes and incubate at 37°C at 150 rpm with the caps on loosely to allow CO_2 ventilation (*see* **Notes 5** and **9**).
5. Take two 1-ml samples at 0, 24, 48, and 72 h for analysis of ethanol and reducing sugars; store samples at −20°C (*see* **Notes 10** and **13**).

3.6. Small-Volume Dilute Acid Pretreatment of Biomass by Autoclaving and Fermentation by Escherichia coli ATCC 53847 (Fig. 1)

3.6.1. Dilute Acid Hydrolysis by Autoclaving

1. Measure 1.5 g dry weight of biomass into 50-ml glass screw-top centrifuge tubes.
2. Add 8.5 ml 1.75%, w/v sulfuric acid.
3. Autoclave the tubes at 121°C for h and allow the tubes to cool to room temperature.
4. Add 1.2 ml $Ca(OH)_2$, 0.55 ml 1 M citric acid, 1.1 ml 10× LB to each tube and adjust the pH to 4.5 (*see* **Notes 2** and **3**).

3.6.2. Enzyme Saccharification

1. Prepare enzymes as directed above (*see* **Subheading 3.3**) for an enzyme loading of 5 FPU cellulase/g dry weight biomass and 60 U cellobiase/g dry weight biomass (*see* **Note 7**).
2. Add enzymes to pretreated biomass tubes and incubate at 37°C at 150 rpm for 24 h (*see* **Notes 5** and **9**).

3.6.3. Inoculation and Fermentation

1. Add 10 µl of 40 µg/ml chloramphenicol stock to each tube.
2. Prepare the inoculum as directed above (*see* **Subheading 3.4.2**) with 1× diluent.

3. Add the inoculum to pretreated and saccharified tubes and incubate at 37°C at 150 rpm with the caps on loosely to allow CO_2 ventilation (*see* **Notes 5** and **9**).
4. Take two 1-ml samples at 0, 24, 48, and 72 h for analysis of ethanol and reducing sugars; centrifuge @ 10,000 × g, filter supernatant and store samples at –20°C (*see* **Notes 10** and **13**).

3.7. Fermentation of Biomass in Bioreactors Using Saccharomyces cerevisiae (Figs. 1 and 2)

3.7.1. Preparation of Bioreactors

1. To each bioreactor, add a magnetic stir bar.
2. To each bioreactor cap, grease largest hole for pH electrode, and place Pasteur pipettes and/or pipetteman tips stuffed with cotton in additional cap holes for ventilation.
3. Place the cap on the bioreactor and cover with a "hat" of aluminum foil.
4. Autoclave at 121°C for 20 min and cool to room temperature.
5. Add 56.7 ml 1.75% (w/v) H_2SO_4 to 10 g dry weight biomass weighed in 250 ml flask. Place a cap on the flask and autoclave separately at 121°C for 1 h. Allow it to cool to room temperature.
6. Transfer the biomass treated with dilute acid in the flask to the autoclaved bioreactor (*see* **Note 4**) and insert pH electrode.
7. Add 15 ml 10% (w/v) $Ca(OH)_2$, 3 ml 1 M citric acid, 20 ml 10× YP to each tube and adjust the pH to 5 (*see* **Notes 2** and **3**).
8. Add distilled water to bring the total volume to 200 ml.

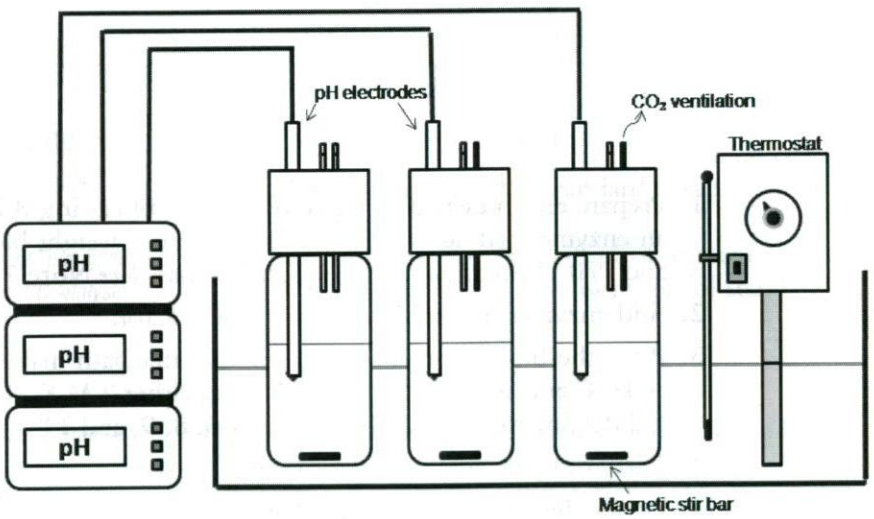

Fig. 2. Schematic of the general equipment used in fermentation of biomass in bioreactors using *S. cerevisiae* and *E. coli* ATCC 53847. *See* **Subheadings 2.1**, **3.7**, and **3.8** for details.

3.7.2. Enzyme Saccharification and Fermentation

1. Prepare enzymes as directed above (*see* **Subheading 3.3**) for an enzyme loading of 5 FPU cellulase/g dry weight biomass and 170 U pectinase/g dry weight biomass (*see* **Note 7**).
2. Add enzymes to the pretreated biomass in the bioreactors.
3. Place the bioreactor in a multi-stirrer water bath maintained at 35°C and stir (*see* **Notes 6**, **8**, **9**, and **11**).
4. Prepare the inoculum as directed above (*see* **Subheading 3.4.1**).
5. Add the inoculum to the above bioreactors with the enzymes to carry out SSF.
6. Take two 1-ml samples at 0, 24, 48, and 72 h to estimate ethanol and reducing sugars centrifuge @ 10,000 × g, filter supernatant and store samples at −20°C (*see* **Notes 10** and **13**).

3.8. Fermentation of Biomass in Bioreactor using Escherichia coli ATCC 53847 (Figs. 1 and 2)

3.8.1. Preparation of Bioreactor

1. To each bioreactor, add a magnetic stir bar.
2. To each bioreactor cap, grease largest hole for pH electrode, and place Pastuer pipettes and/or pipetteman tips stuffed with cotton in additional cap holes for ventilation.
3. Place the cap on the bioreactor and cover with a "hat" of aluminum foil.
4. Autoclave at 121°C for 20 min and cool to room temperature.
5. Add 56.7 ml 1.75% (w/v) H_2SO_4 to 10 g dry weight biomass weighed in 250 ml flask. Place a cap on the flask and autoclave separately at 121°C for 1 h. Allow it to cool to room temperature.
6. Transfer the biomass treated with dilute acid in the flask to the autoclaved bioreactor (*see* **Note 4**) and insert the pH electrode.
7. Add 15 ml 10% (w/v) $Ca(OH)_2$, 3 ml 1 M citric acid, and 40 ml 5× LB to each tube and adjust the pH to 4.5 (*see* **Notes 2** and **3**).
8. Add distilled water to bring the total volume to 200 ml.

3.8.2. Enzyme Saccharification

1. Prepare enzymes as directed above (*see* **Subheading 3.3**) for an enzyme loading of 5 FPU cellulase/g dry weight biomass and 170 U pectinase/g dry weight biomass (*see* **Note 7**).
2. Add enzymes to the biomass in the bioreactor.
3. Place the bioreactor in a multi-stirrer water bath maintained at 45°C and adjust the pH to 4.5 using either 2 M KOH or 2 N HCl; incubate for 24 h (*see* **Notes 6**, **8**, **9**, and **11**).
4. Carry out partial saccharification.
5. Lower the temperature of the water bath to 35°C and adjust the pH to 5.5.
6. Add 200 µl of 40 µg/ml chloramphenicol stock to each bioreactor.

7. Prepare bacterial inoculum as directed above (*see* **Subheading 3.4.2**) and add to the fermentation bioreactor.
8. Take two 1-mL samples at 0, 24, 48, and 72 h to estimate ethanol by GC; store samples at –20°C (*see* **Notes 10** and **13**).

3.9. Gas Chromatographic Analysis of Ethanol Concentration of Fermentation Samples

3.9.1. Prepare GC According to Manufacturers' Directions. Basic Instructions are Provided for the Shimazdu GC-8A (*see* **Note 14**)

1. Open H_2, N_2, and compressed air gas cylinder valves and turn on the GC and the GC integrator.
2. Make sure the GC injector/detector temperature is 250°C and the column temperature is 65°C. The carrier gas (N_2) flow rate should be 0.5 ml/min.
3. Reduce compressed air flow and allow GC to sit for 5 min to accumulate H_2 gas in the column.
4. Ignite the column with a lighter.
5. Return the compressed air flow to the original flow rate and allow the GC to warm up for 15 min.

3.9.2. Prepare Standards for GC Analysis

1. In five individual microcentrifuge tubes, add 50 µl of the 2% (v/v) isopropanol standard solution.
2. Add 50 µl of each standard ethanol solution to the respective tube, mixing well.

3.9.3. Run and Plot Standards for Standard Curve Calculation

1. Inject 1 µl of the 0.5% (v/v) standard solution prepared into the ignited column.
2. Allow ethanol and isopropanol peaks to elute (in that order).
3. Use an integrator or a computer program to record the peak areas for ethanol and isopropanol.
4. Repeat for all standard solutions.
5. Determine the ratio of the ethanol peak to the isopropanol peak for each standard solution: ethanol peak area/isopropanol peak = ratio.
6. Plot ratios (*y*-axis) versus percent ethanol (*x*-axis) to calculate the linear equation of the standard curve.

3.9.4. Prepare Fermentation Samples for GC Analysis

1. For each sample, combine 50 µl of fermentation sample with 50 (–/–) µl of the 2% (v/v) isopropanol standard in a microcentrifuge tube.
2. Mix well.

3.9.5. Run Fermentation Samples

1. Inject 1-µl of the mixed fermentation sample into the ignited GC column.
2. Press "start" on the integrator and wait for the ethanol and isopropanol peaks to elute.
3. Press "stop" on the integrator and record the ethanol and isopropanol peak areas.

3.9.6. Calculate Ethanol Concentrations

1. For each sample, determine the ratio of the ethanol peak area to the isopropanol peak area (*see* **Subheading 3.9.3, step 5**).
2. Using the equation of the line determined in **Subheading 3.9.3, step 6**, determine the percent ethanol of each sample: (ratio – (y-intercept))/slope.
3. To convert percent ethanol to g/l, multiply percent ethanol by 8 [calculation: (x ml ethanol/100 ml H_2O) × (1,000 mL/1 l) × (0.8 g/ml), where (x ml ethanol/100 ml H_2O) is percent ethanol and (0.8 g/ml) is the density of ethanol] (*see* **Note 15**).

4. Notes

1. If the substrate has been frozen, allow it to thaw completely before calculating its percent dry weight. A percent dry weight calculation on a frozen substrate can yield varying results.
2. The volumes of $Ca(OH)_2$ and citric acid may vary. These are used to adjust the pH of the fermentation to the desired value.
3. It is important to take into account the moisture content of the biomass when determining how much growth medium to add to the fermentation vessel. With high moisture content, it may be necessary to add less of a more concentrated growth medium stock to maintain the correct percent solids but provide the needed nutrients to the fermenting organism.
4. The distilled water that needs to be added to the fermentation can be used to rinse out the remaining biomass in the flask from the dilute acid pretreatment to ensure that all the biomass has been transferred to the bioreactor for fermentation.
5. Mixing issues are common for the small-volume dilute acid-pretreated fermentations. This is most common at the beginning of saccharification and/or the beginning of fermentation, leading to incomplete mixing of substances that are added during the course of fermentation. Mixing problems usually correct themselves as saccharification progresses. However, to ensure proper mixing of added liquids, such as acids or bases, the tubes should be agitated and shaken.
6. Stirring issues are very common at the beginning of saccharification and/or the beginning of fermentation; the biomass is often too viscous to be stirred by the magnetic stir bar. This can lead to incomplete mixing and distribution of the enzymes, inoculum, acids, and bases that are added to the fermentation vessel. Most stirring issues correct themselves once the enzymes are added and sufficient time is allowed

to begin the breakdown of the biomass. However, if there is insufficient stirring, the fermentation vessel should be agitated and shaken vigorously to ensure complete mixing when substances are added to the fermentation vessel.

7. Commercial enzyme mixtures are concentrated culture supernatants from enzyme-producing fungi. They are not sterile and should never be autoclaved since the heat would denature the enzymes rendering them inactive. All enzyme mixtures should be filter-sterilized and not added to the fermentation vessel until the vessel has been sterilized separately. Enzymes should be added after the pH is adjusted to the desired value so that they are not denatured.
8. If ethanol is used to disinfect pH probes before administering them into the fermentation apparatus, be sure to thoroughly rinse the probes with sterile water to avoid ethanol contamination.
9. During the course of the fermentation, it is critical to maintain the appropriate temperature and pH that is optimal for the specific step in the process. For the saccharification step, enzymes have different rates of catalysis depending on the temperature and pH, so the indicated temperature and pH are crucial for maximized enzyme activity. Also, the fermenting organism requires a certain pH and temperature in which to grow. If the pH or temperature is too high or too low, this could kill the organism. Either of these scenarios with the enzymes or the organism results in poor ethanol yield.
10. When maintaining pH levels throughout the fermentation, do not extract samples from the fermentation apparatus soon after adding any acid or base. The addition of acid or base just before extracting a sample can interfere with the detection of ethanol yields.
11. Ensure that water bath levels are sufficient throughout the entire fermentation. Many of the fermentations are completed at higher temperatures, resulting in faster evaporation rates. This may cause overheating of the heating element.
12. If conducting replicate fermentations, ensure that each fermentation apparatus is inoculated from a separate liquid culture grown from different colonies.
13. When preparing fermentations for frozen storage, they must be centrifuged and filtered. Centrifugation removes large particle size matter (biomass, organisms) that could clog the filter. Filtering ensures that the fermenting organism is removed from the sample, preventing any further conversion

of sugars to ethanol. It is also very important that all samples that are to be analyzed by GC be filtered. This prevents fouling of the column or detector. In addition, it is critical to store samples in tubes that prevent evaporation of ethanol during storage.

14. The GC septum should be changed every time the GC is operated.
15. If antibiotics that have been dissolved in ethanol are added to the fermentation apparatus before inoculation, correct the apparent yields during the fermentation for the ethanol present.
16. These different methodologies may result in differing ethanol yields depending on the biomass source, composition of the biomass, and/or the substrate range of the fermenting organism. Another consideration is that Dilute Acid Hydrolysis (DAH) pretreatment may be ineffective depending on the substrate (production of inhibitory compounds, release of heavy metal, etc.). *See* **Fig. 3** an example of fermenting the same mixed carbohydrate biomass containing 5C and 6C sugars for yeast and bacteria using the small-volume fermentation protocol compared to the bioreactor protocol. These fermentations have not been optimized to increase ethanol yields.

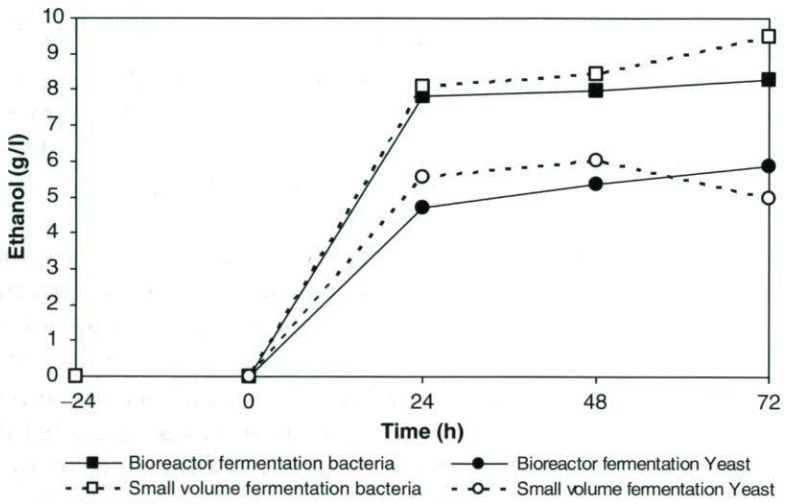

Fig. 3. Ethanol yields from the fermentation of forest residue biomass by two methods using *S. cerevisiae* (*circles*) and *E. coli* ATCC 53847 (*squares*) in small-volume DAH and fermentations (*dashed lines*) and bioreactor DAH and fermentation (*solid lines*). The 24-h time point corresponds to the beginning of the 24-h saccharification step for the bacteria only (PSCF). Yeast fermentations were true SSF without a preincubation step. Inoculation occurred at time 0 h.

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